REMARKS

Reconsideration of the present application is respectfully requested.

Claims 75-115 are pending in the application. Claims 75-78 are withdrawn. Claims 79-115 have been newly added. Support for new claims can be found throughout the specification, e.g., page 25, lines 13-17; original claims; and in the earliest parent application (Ser. No. 09/041,329), e.g., par. Spanning pages 17-18. No new matter was added.

Request for Personal Interview

Due to the complexity of the subject matter and legal issues involved, pursuant to 37 CFR 1.133, Applicants' Attorney, Nada Jain (undersigned) respectfully requests a Personal Interview with the Examiner and the Examiner's Supervisor at the Examiners' convenience to discuss the patentability of the present claims. While Applicants appreciate the Examiner's knowledge of the case, in the interest of thorough, quality examination, Applicants welcome the presence of the Examiner's Supervisor. Please telephone the undersigned to schedule the Interview.

Rejection under 35 USC Section 103

Claims 31-74 stand rejected as being obvious over Romanczyk, Jr. (US 5,554,645; hereinafter referred to as 'Romanczyk') in view of Wideman *et al.* (US 6,127,421; hereinafter referred to as 'Wideman') on the ground that a person of skill in the art would have been motivated to combine compounds/compositions of Romanczyk and Wideman because both are taught to be useful for the same purpose (anti-tumor) to form a third composition used for the same purpose.

To expedite the prosecution of the present application, Applicants file herewith a Request for Continued Examination together with evidence of unexpected benefits of the invention recited in new claims. Claims 31-74 have been canceled and the rejection is

moot with respect to them. Applicants address the rejection with respect to the new claims.

The composition recited in the present claims is patentable over the combined teaching of Romanczyk and Wideman because of its unexpected benefits as explained below:

In mammals, L-arginine is a common substrate for both: (i) nitric oxide synthase (NOS), which catalyzes the synthesis of nitric oxide (NO) and citrulline, and (ii) arginase, which catalyzes the hydrolysis of arginine to ornithine and urea. NO is produced by endothelium and has a smooth-muscle relaxing effect resulting in vasorelaxation of blood vessels and increased blood flow which are important for healthy vascular function. The availability of L-arginine can be a rate-limiting factor for cellular NO production by NOS because arginase competes with NOS for L-arginine utilization. *See e.g.* abstract, background and discussion *in* Schnorr *et al.*, Arch. Biochem. Biophys., 476: 211-215 (2008) (Attachment 1). Decreased synthesis or bioavailability of NO is a hallmark of endothelial dysfunction, which is an early event in the pathogenesis of cardiovascular diseases; the competition from vascular arginase for the common substrate L-arginine may impair NO production even when there is appropriate NOS activity (Attachment 1, page 211, par. spanning cols. 1-2). Thus, decreasing arginase expression and/or activity would beneficially contribute to improved L-arginine availability for NO production.

Compositions recited in Applicants' claims have such an unexpected benefit, *i.e.*, they decrease arginase expression and activity. This is because cocoa polyphenol and the polyphenolic compound of formula An recited in Applicants' claims beneficially modulate NO (*see* specification, e.g., Examples, pages 58-68) and are also effective in reducing gene expression and activity of arginase (*see* both *in vivo* and *in vitro* data, including human data, presented in Attachment 1, *e.g.* Figures 1-3 and Table 3). In other words, in the presence of cocoa polyphenol/polyphenol of formula An, more L arginine becomes available for NO production due to reduced competition from arginase. This

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unexpected benefit is neither disclosed nor suggested by the cited prior art; withdrawal of the rejection is respectfully requested.[1]

* * *

With respect to the Examiner's statements made in Examiner's Answer Applicants wish to point out that the Examiner focused on a motivation to combine but failed to address the second prong of obviousness analysis, reasonable expectation of success. The Supreme Court's decision in *KSR International Co. v. Telefex Inc.*, 550 U.S. _, 82 U.S.P.Q.2d 1385 (2007), while binding, did not change the long standing legal requirement for showing reasonable expectation of success. In *KSR*, the Supreme Court "reaffirmed the familiar framework for determining obviousness as set forth in *Graham v. John Deere Co.*, but stated that the Federal Circuit had erred by applying the teaching-suggestion-motivation (TSM) test in an overly rigid and formalistic way." *Examination Guidelines for Determining Obviousness Under 35 U.S.C. 103 in View of the Supreme Court Decision in KSR International Co. v. Teleflex Inc.*, 72 Fed. Cir. 57526, par. bridging cols. 2-3, [hereinafter "Guidelines"] citing *KSR*, 82 USPQ2d at 1391.[2] The issue in *KSR* was obviousness of a mechanical invention which represented a combination of elements found in the prior art. The Supreme Court recognized that predictability was a necessary component of obviousness analysis and stated that a combination of known elements would have been obvious if "the combination would have yielded nothing more than predictable results to one of ordinary skill in the art at the time of the invention." *KSR*, 82 USPQ2d at 1395 (emphasis added); *see also* Guidelines at 57529, 2nd col.

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[1] The unexpected benefit of the compositions recited in Applicants' claims makes the compositions particularly useful for cardiovascular applications. In contrast, administering the compositions to subjects suffering from cancer may be harmful because increased NO can increase tumor angiogenesis, increase metastatic potential of cancer, disable apoptotic process (*e.g.*, by nitrosylation of caspase) and can cause nitrosylation of growth factor receptors, a key step in many cancers. Should the Examiner find any background information helpful, Applicants can provide scientific articles containing such information during the Interview.

[2] In the interest of conservation, Applicant does not provide copies of *KSR* or Guidelines as they are likely to be readily available to the Examiner. Should the Examiner require a copy, please telephone the undersigned.

On July 21, 2008, ruling on obviousness of a chemical invention, the Federal Circuit Court applied the holding of *KSR* to chemical inventions. *Eisai Co. v. Dr. Reddy's Labs., Ltd*, 2007-1397, -1398; 2008 U.S. App. LEXIS 15399.[3] The Federal Circuit Court recognized that *KSR* contemplated instances where "some reasons for narrowing the prior art universe to a 'finite number of identified, predictable solutions'" could exist but stated that chemical arts are often unpredictable and "KSR's focus on these 'identified, predictable solutions' may present a difficult hurdle because potential solutions are less likely to be genuinely predictable." *Eisai*, page 8.

Accordingly, based on *KSR*, as explained by *Eisai*, to be obvious, the present claims must have been predictable from the combined teachings of cited references, *i.e.*, a person of skill in the art would have had to reasonably expect that a composition useful for treating cancer could have been obtained by combining the teachings of the cited references. "[P]rior art must be considered in its entirety, including disclosures that teach away from the claims" MPEP, Section 2141.02 VI. "When prior art contains apparently conflicting references, the [Office] must weigh each reference for its power to suggest solutions to an artisan of ordinary skill." *In re Young*, 927 F.2d 588, 591; 18 U.S.P.Q.2d 1089 (Fed. Cir. 1991). In addition to the evidence of record, Applicants enclose herewith a lecture by Kenneth Park entitled "The Immunological and Metabolic Effects of L-arginine in Human Cancers" (Attachment 2, Proc. Nutrit. Soc. 52,387-401 (1993)), in which studies in animals and humans with cancer showed that L-arginine supplementation stimulated tumor growth and tumor protein synthesis, respectively. At the Interview, Applicants' representative will present for discussion a number of additional publications describing studies with L-arginine in the context of cancer. Based on this information, a person of skill in the art would have doubted that a composition effective for treating cancer could have been obtained by combining cocoa polyphenol/polyphenol of formula An and L-arginine in the amounts recited in the present claims.

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[3] In the interest of conservation, Applicant does not provide a copy of *Eisai* since it is likely to be readily available to the Examiner. Should the Examiner require a copy, please telephone the undersigned.

* * *

With respect to the Examiner's statement made in Examiner's Answer that the cocoa procyanidin/polyphenol of formula An taught by Romanczyk and arginine taught by Wideman can be optimized for NO effects (even though such are not suggested by the cited art) "because the instant specification provides for very broad amount ranges of the instantly claimed ingredients therein" and further that

> the amounts of cocoa polyphenol and arginine disclosed by the respective reference teachings (*i.e.*, Romanczyk discloses high levels/percentages of cocoa polyphenol within such non-chocolate food compositions for inhibiting tumor growth; and Wideman discloses the prior art use of an anti-tumor feed composition comprising 0.92% or 2.40% of arginine therein) would intrinsically provide the instantly claimed *in vivo* functional effect with respect to increasing nitric oxide, upon ingestion thereof

Examiner's Answer, page 9, Applicants note the following.

It is well established under the US Patent Law that only result-effective variables can be optimized. MPEP Section 2144.05 II B. "A particular parameter must first be recognized as a result-effective variable, i.e., a variable which achieves a recognized result, before the determination of the optimum or workable ranges of said variable might be characterized as routine experimentation." *Id.* This is not the case here because the cited prior art fails to teach that the compounds cited in Applicant's claims have nitric oxide effect; hence, in the absence of such a recognized result, arriving at the above quoted structural limitation of Applicants claims would not have amounted to routine optimization. It appears that the Examiner believes that the amounts recited in the present claims are the same as those recited in the cited art. However, the combined amounts recited in the present claim are not identically disclosed in the cited art and a person of skill in the art would have had to conduct experimentation to arrive at them.

Moreover, claims 109 to 115, contain an entirely different range of L-arginine than disclosed in Wideman.

* * *

In view of the above amendments and remarks, withdrawal of the rejection is believed to be in order. An action to that effect is respectfully requested.

CONCLUSION

Applicants believe that the application is now in condition for allowance. A notice to that effect is respectfully requested.

Respectfully submitted,

Date: December 16, 2008

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Attachment 1

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Cocoa flavanols lower vascular arginase activity in human endothelial cells *in vitro* and in erythrocytes *in vivo*

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ABSTRACT

The availability of L-arginine can be a rate-limiting factor for cellular NO production by nitric oxide synthases (NOS). Arginase competes with NOS for L-arginine as the common substrate. Increased arginase activity has been linked to low NO levels, and an inhibition of arginase activity has been reported to improve endothelium-dependent vasorelaxation. Based on the above, we hypothesized that an increase in the circulating NO pool following flavanol consumption could be correlated with decreased arginase activity. To test this hypothesis we (a) investigated the effects of (−)-epicatechin and its structurally related metabolites on endothelial arginase expression and activity *in vitro*; (b) evaluated the effects of dietary flavanol-rich cocoa on kidney arginase activity *in vivo*; and (c) assessed human erythrocyte arginase activity following flavanol-rich cocoa beverage consumption in a double-blind intervention study with cross-over design. The results demonstrate that cocoa flavanols lower arginase-2 mRNA expression and activity in HUVEC. Dietary intervention with flavanol-rich cocoa caused diminished arginase activity in rat kidney and, erythrocyte arginase activity was lowered in healthy humans following consumption of a high flavanol beverage *in vivo*.

The flavan-3-ol (−)-epicatechin is, at least in part, causally linked to improved vascular function in humans following the consumption of a high-flavanol cocoa [1]. Ingestion of a cocoa drink high in flavanols results in an increase in the circulating nitric oxide (NO)[1] pool that is paralleled by a L-NMMA-sensitive augmentation in dilation of the brachial artery [1–3]. A mechanistic interpretation of the above data is that circulating flavanols (and/or their metabolites) may increase endothelial nitric oxide synthase (eNOS)-dependent NO production, which mediates the observed augmentation in arterial dilation. Most potential explanations for such increases in eNOS-derived NO levels involve modulation in either eNOS activity, a change in eNOS substrate availability or enhanced NO levels via inhibition of NADPH oxidase [4,5]. Thus, L-arginine metabolism becomes of interest.

In mammals, arginase exists in two isoforms, both catalyzing the conversion of L-arginine to urea and L-ornithine [6]. Arginase-1, a protein that is elevated in inflammatory diseases, is located in the cytosol, and it is mainly expressed in liver and macrophages [7]. Arginase-2 is primarily expressed in extrahepatic tissues, with high abundance in the kidney. In the context of the above, the synthesis of NO through oxidation of L-arginine by NOSs is important for vascular homeostasis. Decreased synthesis or bioavailability of NO is a hallmark of endothelial dysfunction, an early event in the pathogenesis of cardiovascular diseases. The availability of L-arginine for the eNOS-catalyzed NO synthesis can represent a rate-limiting factor in cellular NO production *in vivo* and in *vitro* [8,9]. Vascular arginase competes with eNOS for their common substrate L-arginine, and thus it may impair NO production even when there is appropriate eNOS activity [10]. Recent findings provided evidence for a causal link between endothelial arginase activity, eNOS-dependent NO production and vascular dysfunction, in the context of arteriosclerosis [11,12], hypertension [13,14], age-associated cardiovascular disease [15,16], and ischemia reperfusion-induced loss of arterial function [17].

Based on the above we hypothesized that flavanols might decrease endothelial and erythrocyte arginase activity contributing to improved L-arginine availability inside the vessel. To test this hypothesis we investigated the effects of (−)-epicatechin and its main structurally related metabolites on endothelial cell arginase expression and activity *in vitro* using human umbilical endothelial cells (HUVEC) and evaluated the effects of dietary flavanol-rich cocoa on kidney arginase activity *in vivo*. Furthermore, we assessed

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[1] *Abbreviations used:* NO, nitric oxide; eNOS, endothelial nitric oxide synthase; HUVEC, human umbilical endothelial cells; ISPF, α-isonitrosopropiophenone; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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human erythrocyte arginase activity following the consumption of a flavanol-rich cocoa beverage in a double-blind human intervention study with cross-over design.

Materials and methods

Materials

Chemicals were purchased from Sigma (Deisenhofen, Germany) except when stated otherwise. The flavanol metabolite mixture provided a sum of total flavanols of 2.6 μM, consisting of (−)-epicatechin (0.1 μM) and catechin (0.4 μM) as well as the flavanol metabolites, epicatechin-7-β-D-glucuronide (0.25 μM), 4′-O-methyl-epicatechin (0.2 μM), and 4′-O-methyl-epicatechin-7-β-D-glucuronide (1.7 μM) and was dissolved in ethanol. The above concentrations are equivalent to the average plasma concentration of these compounds that were measured in healthy human subjects 2 h after they consumed 200 ml of a high-flavonoid cocoa beverage that provided 985 mg of flavonoids as described recently [1].

Study protocols

Cell culture work

HUVEC were purchased from Promo Cell, (Heidelberg, Germany) and were cultured in a customer-formulated, nitrite- and nitrate-free medium (Promo Cell) for up to two passages. Cell culture experiments were performed with 5×10^5 cells per 6-cm dish.

Human subjects

The effects of ingestion of a high-flavanol versus a low-flavanol cocoa beverage on erythrocyte arginase activity were investigated in a group of 10 healthy volunteers in a double-blind study with cross-over design. Exclusion criteria were smoking, hypertension, diabetes mellitus and renal failure. On 2 days we measured arginase activity in erythrocytes before, 2 and 24 h after the ingestion of either a high-flavanol (985 mg) or a low-flavanol (<90 mg) drink (for details see Table 1; cocoa powder was kindly provided by Dr. C. Kwik-Uribe, Mars Inc., Hackettstown, NJ, USA). Individuals were studied in the morning after a 12-h fasting period. To avoid accumulation effects, investigations were separated by a wash out phase of at least 6 days. The study was approved by the Ethics Board of the Medical Faculty of the Heinrich-Heine-University Duesseldorf.

Animal studies

Ten male Sprague–Dawley weanling rats were obtained from Charles River Laboratories (Wilmington, MA, USA). Rats were individually housed in suspended stainless-steel cages in a temperature (23 °C) and photoperiod (14–10 h light–dark cycle) controlled room. They were divided into two groups ($n = 5$ per group) after a 6-day adaptation period and given stock diet for 2 days, ad libitum, followed by control diet for 4 days, with meal feeding (5 h/day). The animals were then randomly assigned to one of two diet groups and were given restricted access (5 h/day during dark cycle: 12 am–5 am) to purified egg white protein-based diet containing 59.5% fructose with either 0% or 4% cocoa powder for 28 days. Consumption of high-fructose diets is associated with an increase in tissue oxidative stress [19]. High-fructose diets are now being commonly used for studies of liver disease and vascular disease [20,21]. The amount of cocoa used in the current study was based on previous work that demonstrated that, for rats, the chronic consumption of diets containing this amount of cocoa is associated with reductions in markers of oxidative damage [22] others use similar diets [23]. Both groups were allowed free access to water delivered via a stainless-steel watering system. The detailed composition of the diets is shown in Table 2. The cocoa used for this study was especially high in flavanols and procyanidins and contained 11.0 mg epicatechin/g, 2.8 mg catechin/g, and 43.0 mg procyanidins/g.

At the end of the 28-day period, rats were euthanized after a 2-h fast. The animals were anesthetized with carbon dioxide, and kidneys were quickly excised, weighed, and stored at −80 °C until analysis.

Experimental protocols were approved before implementation by the Animal Use and Care Committee at the University of California, Davis, and were administered through the Office of the Campus Veterinarian.

Quantitative gene expression analysis

Real-time PCR on the LightCycler® (Roche Diagnostics, Mannheim, Germany) was performed in a total volume of 20 μl containing 2 μl cDNA, 2 μl Fast Start Reaction Mix SYBR Green I, 1.6 μl of 25 mM $MgCl_2$, 2 μl of each primer 5 pmol/μl and 10.4 μl H_2O. For negative controls, the same RNA preparations were used with the omission of the reverse transcriptase step. After completion of the cycling process, samples were subjected to a temperature ramp with continuous fluorescence monitoring for melting curve analysis. For each PCR product, apart from primer-dimers, a single narrow peak was obtained by melting curve analysis at the specific melting temperature, and only a single band of the predicted size was observed by agarose gel electrophoresis, indicating specific amplification without significant byproducts. Samples were quantified accordingly (LightCycler® analysis software, version 3.5) using the housekeeping gene GAPDH as standard.

Arginase activity

Erythrocytes from venous blood were separated by differential centrifugation, and arginase activity was determined immediately [18]. HUVEC cells were lysed and incubated with 10 mM $MnCl_2$ at 55 °C for 10 min. Then one volume 0.5 M L-arginine was added and incubated for 60 min at 37 °C. The reaction was stopped by adding an 800-μl acid mixture ($H_2SO_4/H_3PO_4/H_2O$, 1:3:7), and urea was quantified colorimetrically at 540 nm after the addition of 50 μl 9% α-isonitrosopropiophenone (ISPF) and heating at 96 °C for 45 min. The amount of urea produced, after normalization with protein, was used as an index for arginase activity. For the determination of protein concentration in erythrocytes, lysates were diluted 1:100.

Arginase activity was determined in kidney homogenates prepared from frozen tissue segments according to a modified method [24] of Brown and Cohen [25].

Table 1
Composition of the cocoa powder used to prepare the beverages (54 g/200 ml of water)

Ingredient	High-flavanol (units/serving)	Low-flavanol (units/serving)
Energy (kJ)	666	717
Total fat (g)	3.0	3.0
Sodium (mg)	180	420
Total carbohydrates (g)	27.0	27.0
Fiber (g)	12.0	12.0
Sugar (g)	15.0	15.0
Protein (g)	15.0	15.0
Caffeine (mg)	31.8	36.6
Theobromine (mg)	585	570
Total cocoa flavanols (mg)	985	80.4
Epicatechin (monomer) (mg)	183	19.8
Catechin (monomer) (mg)	61.2	4.8
Dimer (mg)	215	23.1
Trimer (mg)	174	14.6
Tetramer (mg)	152	12.0
Pentamer (mg)	102	6.3
Hexamer (mg)	53.7	0
Heptamer (mg)	34.5	0
Octamer (mg)	5.7	0

Table 2
Diet composition

Ingredient (g/kg diet)	Control	Cocoa (4%)
Egg white[a]	210	210
Corn starch[b]	0	0
Fructose[c]	595	595
Corn oil	80	80
Mineral mix[d]	60	60
Alphacel[e]	40	40
High biotin vitamin mix[f]	15	15
Cocoa power[g]	0	40

[a] Spray-dried egg white was obtained from Dyets Inc., (Bethlehem, PA, USA).
[b] Corn starch was obtained from National Starch and Chemical Co., (Bridgewater, NJ, USA).
[c] Fructose was obtained from ICN Biomedicals Inc., (Aurora, OH, USA).
[d] Mineral mix contained the following (g/kg mix): $CaCO_3$, 139.7; $CaHPO_4$, 188.6; K_2HPO_4, 133.6; NaCl, 21.2; $MgSO_4$, 49.5; $FeSO_4 \cdot 7H_2O$, 6.2; $ZnCO_3$, 0.8; $MnSO_4 \cdot H_2O$, 0.61; $CuSO_4 \cdot 5H_2O$, 0.66; KI, 0.0033; $CrK(SO_4)_2 \cdot 12H_2O$, 0.048; Na_2SeO_3, 0.015; $Na_2MoO_4 \cdot 2H_2O$, 0.0063, Cerelose 481.
[e] Alphacel, nonnutritive bulk, was obtained from INC Biomedicals Inc. (Aurora, OH, USA).
[f] Vitamin mix (g/kg mix): Inositol, 25.0; ascorbic acid, 5.0; calcium pantothenate, 0.67; thiamine hydrochloride, 0.27; pyridoxine hydrochloride, 0.53; nicotinine acid, 1.0; menadione, 0.25; riboflavin, 0.27; p-aminobenzoic acid, 0.50; folic acid, 0.067; biotin, 0.26; all-rac-α-tocopherol, 1.20; retinol, 0.047; cholecalciferol 0.0017; vitamin B_{12} + mannitol, 3.33; choline chloride (70% sol mL/kg), 71.50; cerelose, 887.
[g] Cocoa powder was obtained from Mars Inc. (Hackettstown, NJ, USA) it contained per g: 13.8 mg monomer including 11.0 mg epicatechin and 2.8 mg catechin, 10.5 mg dimer, 7.7 mg trimer, 6.7 mg tetramer, 5.1 mg pentamer, 4.2 mg hexamer, 2.3 mg heptamer, 2.1 mg octamer, 3.2 mg nonamer, and 1.2 mg decamer.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity

Frozen kidney segments were homogenized in 10 volumes of ice-cold buffer (10 mM Tris, pH 7.4, 0.25 M sucrose, 0.5 mM EDTA) and centrifuged at 10,000g for 30 min at 4 °C. GAPDH activity in the supernatants was measured according to Bergmeyer [26].

Statistical analyses

Values are reported as means ± SD. Statistical analysis was made by unpaired Student's t-test for cell culture experiments and paired Student's t-test for the human study. For both tests $p < 0.05$ was considered significant. Analyses were calculated with Excel 2003.

Results

(−)-Epicatechin and its structurally related metabolites lower arginase-2 mRNA expression and arginase activity in HUVEC

Arginase-2 mRNA expression levels were investigated in human endothelial cells using real-time PCR. The composition of the flavanol metabolite mixture, and the concentrations of the individual flavanols, used for this *in vitro* study were equivalent to the average flavanol concentrations found in human plasma [1] 2 h after subjects consumed a high-flavanol cocoa beverage similar to the one used in the current study. Cells cultured in media containing the flavanol metabolite mixture were characterized by lowered arginase-2 mRNA expression compared to control cells. The decrease, detectable by 2 h, was more pronounced at 24 h (Fig. 1). Consistent with the results obtained with the flavanol metabolite mixture, the addition of (−)-epicatechin also led to decreased arginase-2 mRNA expression after 24 h of incubation. In contrast to arginase-2 mRNA, a weak basal arginase-1 mRNA expression was not affected by either (−)-epicatechin or the flavanol metabolite mix (data not shown).

No effect of flavanols on arginase activity was observed at 2 and 24 h (data not shown), but at 48 h arginase activity in control cells was 0.69 ± 0.04 μmol urea mg protein^{-1} h^{-1}, whereas in cells treated with 10 μM (−)-epicatechin activity was lowered down to 0.46 ± 0.05 μmol urea mg protein^{-1} h^{-1} (Fig. 2). This is a decrease comparable to the response of cells challenged with a mixture of the proinflammatory cytokines IL-1β and TNF-α (0.37 ± 0.07 μmol urea mg protein^{-1} h^{-1}), known to decrease arginase expression [27]. Similarly, cells incubated with the mixture of flavanol metabolites exhibited lowered arginase activity in a dose-dependent manner.

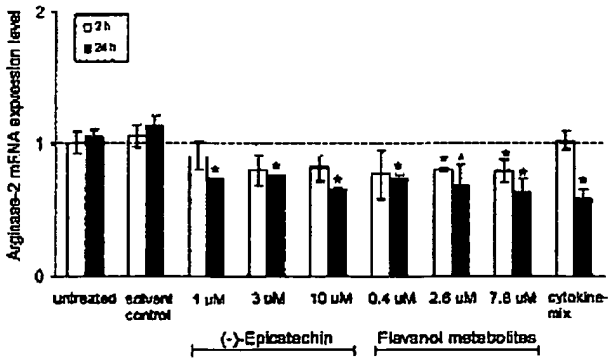

Fig. 1. Flavanols decrease expression of arginase-2 mRNA in human endothelial cells. HUVEC were cultured in the presence of (−)-epicatechin or conjugated flavanol metabolites for 2 and 24 h. RNA was extracted, reversely transcribed and amplified by real-time PCR. Relative expression was calculated and the results normalized to cDNA template concentration. (−)-Epicatechin, as well as its structurally related metabolites, led to lowered arginase-2 mRNA expression after 24 h compared to control levels. As a positive control, a cytokine mix (TNF-α + IL-1β, each 500 U/ml) also decreased arginase-2 mRNA expression. An early response was observed after 2 h of incubation in presence of 2.6 and 7.8 μmol/L flavanol metabolites. Bars represent the means of three individual experiments ± SD. For each experiment triplicates were performed. $^{*}p < 0.05$ vs. solvent control.

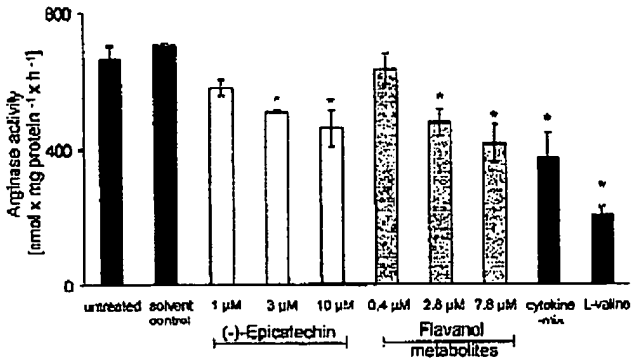

Fig. 2. Flavanols lower activity of arginase in human endothelial cells. HUVEC were cultured in the presence of epicatechin or conjugated flavanol metabolites mixture for 48 h. Then, cells were lysed and arginase activity was assayed. (−)-Epicatechin and conjugated flavanol metabolites led to dose-dependent inhibition of vascular arginase activity. A cytokine mix (TNF-α + IL-1β, each 500 U/ml) known to decrease arginase expression and activity, was used as a positive control. Treatment with L-valine (10 mM), as an arginase inhibitor was used as control. Bars represent the means of four individual experiments ± SD. $^{*}p < 0.05$ vs. solvent control.

High-flavanol containing cocoa-based diet diminishes renal arginase activity in rats in vivo

Further evidence on flavanol-mediated effects on arginase activity was provided from an animal experiment. Rats were fed a diet containing 59.5% (w/w) fructose as carbohydrate source with or without 4% (w/w) flavanol-rich cocoa (Table 2), containing (−)-epicatechin and its oligomers as the major cocoa flavanols. As arginase-2 is most abundantly expressed in kidney, we investigated the effect of flavanols on renal arginase activity. This dietary intervention with high-flavanol cocoa resulted in lowered renal arginase activity, whereas GAPDH activity as a control was not affected (Table 3).

Consumption of flavanol-rich cocoa results in a decrease in erythrocyte arginase activity in healthy human adults

Arginase activity was assayed in erythrocytes before, 2 and 24 h after the ingestion of 200 mL of either a high-flavanol, or a low-flavanol cocoa drink. Consumption of the high flavanol-containing cocoa drink was associated with a decrease in erythrocyte arginase activity after 24 h (3.0 ± 0.3 μmol urea mg protein^{-1} h^{-1}; $p < 0.05$) compared to controls (3.9 ± 0.4 μmol urea mg protein^{-1} h^{-1}) (individual values are shown in Fig. 3). In contrast, the ingestion of the low-flavanol cocoa drink did not lead to a lowered enzyme activity (3.7 ± 0.4 vs. 3.5 ± 0.5 μmol urea mg protein^{-1} h^{-1}; p = ns). Two hours after ingestion neither the high-flavanol nor the low-flavanol cocoa did change arginase activity (data not shown).

Table 3
Cocoa powder-containing diet lowers arginase activity in rat kidney

Enzyme (U/mg protein)	Control	Cocoa (4%)
Arginase	0.18 ± 0.02	0.13 ± 0.02*
GAPDH	1.30 ± 0.10	1.25 ± 0.11

Rats fed with a casein-based diet for 28 days containing fructose (59.5% (w/w)) as carbohydrate source with or without cocoa (4% (w/w)) (see Table 2). Data are given as means ± SD.
$^{*}p < 0.05$.

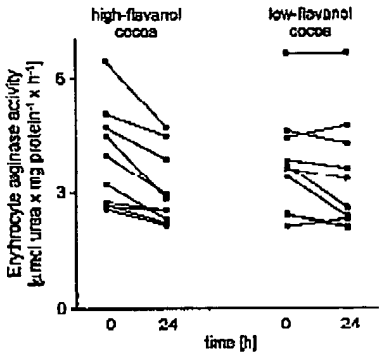

Fig. 3. High-flavanol cocoa lowers erythrocyte arginase activity in healthy humans. Arginase activity was assayed in erythrocytes of healthy volunteers ($n = 10$) before ($t = 0$ h) and 24 h after the ingestion of 200 ml of a cocoa drink high, or low, in flavanols (Table 1). Erythrocyte arginase activity was diminished by the high, but not by the low flavanol cocoa beverage at 24 h. Black squares represent individual values. Mean values ± SEM in [µmol urea mg protein^{-1} h^{-1}] are: high-flavanol cocoa at $t = 0$ h: 3.9 ± 0.3; and at $t = 24$ h: 3.0 ± 0.4; $^{*}p < 0.05$ Low-flavanol cocoa at $t = 0$ h: 3.7 ± 0.4; and at $t = 24$ h: 3.5 ± 0.5; p = ns.

Discussion

Regulation of L-arginine metabolism

Results from acute dietary intervention studies have suggested that flavanols can provide beneficial cardiovascular effects as a consequence of a flavanol-induced increase in the circulating NO pool as reflected by improvement in endothelial function (see [13]). Cardiovascular homeostasis is, at least in part, NOS-dependent, with L-arginine availability being a potential rate-limiting factor in endothelial NO synthesis. Arginase expression contributes to the regulation of intracellular L-arginine concentration, and increases in arginase protein expression are associated with a number of pathophysiological conditions such as inflammation [28] or wound healing [29], resulting in local L-arginine deprivation in the tissue. This concept is further supported by recent findings that causally link diminished plasma L-arginine concentrations in inflammatory diseases to increased arginase activity and decreased bioavailability for NOS-derived NO [30,31]. This can subsequently lead to a lowered NO synthesis, which in blood vessels may contribute to the initiation and progression of atherosclerosis. Erythrocytes represent an important compartment for NO metabolism and synthesis in the blood, as plasma nitrite (NO_2^-) can be oxidized to nitrate (NO_3^-) in a hemoglobin-dependent manner and, in addition, erythrocytes express a functionally active form of eNOS [32]. Interestingly, increased erythrocyte arginase activity was reported in patients with sickle cell disease, and was associated with lowered plasma NO levels and with impairment in both erythrocyte and vascular function [33]. The results presented here demonstrate that the consumption of a flavanol-rich beverage leads to a statistically significant decrease in erythrocyte arginase activity (Fig. 3). Thus, this study links dietary flavanol intake and arginase-mediated L-arginine turnover to the established effects of flavanols on improved NO bioavailability and circulating NO pools [34].

Lowered arginase expression and activity in vitro and in vivo

Consistent with the results obtained with humans (Fig. 3), *in vitro* experiments with HUVEC treated with either (−)-epicatechin, or its structurally related metabolites in concentrations attainable in humans, demonstrated a lowered arginase-2 mRNA expression (Fig. 1) and arginase activity (Fig. 2). In addition, the effect of flavanols on arginase activity was replicated in an animal dietary intervention study as a high-flavanol diet results in a diminished renal arginase activity (Table 3). These results suggest that cocoa flavanols have different effects on the L-arginine metabolism in mammals. First, an acute short-term effect (30 min to 1–2 h), mediated by up-regulated NO synthesis resulting in a increased FMD, whereas a second, longer-term effect (24 h or longer) of flavanols involves the down-regulation of arginase gene expression and, which might lead as a consequence thereof, to decreased enzyme activity. Of course, other mechanisms like CAT-mediated L-arginine import, a temporarily depleted L-arginine pool as result of increased NO synthesis or interaction of flavanols with other enzymes, like NADPH-oxidases might contribute to the regulation of flavanol-dependent eNOS/arginase mediated L-arginine turnover. Repeated ingestion of high-flavanol cocoa over 7 days led to an increase in baseline FMD reaching a plateau at day 5 and enhanced nitrite plasma concentration [35]. This sustained increase of NO-dependent vascular parameters may, at least in part, a result of diminished arginase activity.

As erythrocytes do not have nuclear gene activity, there has to be another mechanism of action on L-arginine metabolism in the erythrocyte. A variety of explanations for lowered erythrocyte arginase activity are conceivable, as (−)-epicatechin may mediate alterations in L-arginine import by cationic amino acid transporters, decreased arginase mRNA or protein stability or improved erythrocyte-NOS activity, which can lead to a lowered L-arginine pool.

In summary, the results presented here provide evidence for a flavanol-mediated decrease of arginase expression and activity *in vitro* and *in vivo*, suggesting a contribution to the regulation of intracellular L arginine concentration and NOS substrate supply. These findings may provide new opportunities for dietary or therapeutic interventions to regulate L-arginine and NO bioavailability.

Acknowledgments

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Attachment 2

Proceedings of the Nutrition Society (1993), **52**, 387–401

PROCEEDINGS OF THE NUTRITION SOCIETY

A Scientific Meeting was held at Regent's College, London on 2/3 December 1992

The Sir David Cuthbertson Medal Lecture 1992

The immunological and metabolic effects of L-arginine in human cancer

BY KENNETH G. M. PARK

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It is both a great pleasure and an honour to be able to deliver this third Cuthbertson lecture, in memory of course of Sir David Cuthbertson who was, very much, the founding father of clinical metabolism. The work which I will describe in the present lecture was performed both at the Rowett Research Institute in Aberdeen and at the University Department of Surgery, also in Aberdeen. Cuthbertson had close connections with Aberdeen and was a former director of the Rowett Research Institute, 22 years previously, a post which he held from 1945 to 1965. This was obviously before my time in Aberdeen and in fact my only encounter with Sir David was as a teenager, when he threw me off Royal Troon golf course.

In Aberdeen my function was rather like that of a cytokine, linking the metabolic work at the Rowett with the immunological work at the Department of Surgery, in looking at the effects of nutrition on cancer growth and metabolism.

BACKGROUND

The introduction of modern enteral and parenteral feeding regimens into clinical practice has meant that it is now possible to provide nutritional support to patients in a wide variety of clinical settings. In surgical practise the consequences of malnutrition (both obesity and undernutrition) are only too familiar to most clinicians. For example, many authors have reported diminished immunity, poor wound healing and reduced voluntary muscle activity in undernourished patients (Von Meyenfeldt *et al.* 1988; Windsor & Hill, 1988; Wan *et al.* 1989). Studley (1936) first showed this to represent a significant risk to the surgical patient, and since then many other authors have confirmed the relationship between undernutrition and increased surgical morbidity, and even mortality (Conti *et al.* 1977; Muller *et al.* 1986; Detsky *et al.* 1987). Despite these observations it is not at all clear whether pre- and/or peri-operative nutrition can reverse malnutrition and reduce morbidity in such patients (Veterans Affairs Total Parenteral Nutrition Cooperative Study Group, 1991). There is even more conjecture regarding the value of nutritional support in malnourished cancer patients. A number of prospective controlled trials have been conducted (Table 1).

Care must be exercised in evaluating these trials. Comparisons are frequently made between groups containing both malnourished and well-nourished patients, assuming

Table 1. *Summary of the results of the trials of preoperative nutritional support in patients with cancer*

Source	No. in each group		Mortality		Morbidity	
	Supplement	No supplement	Supplement	No supplement	Supplement	No supplement
Holter & Fisher (1977)	30	26	2	2	11	11
Moghissi *et al.* (1977)	10	5	—	—	0	1
Heatley *et al.* (1979)	38	36	6	8	5	14
Sako *et al.* (1981)	35	33	3	0	15	18
Thompson *et al.* (1981)	12	9	0	0	5	3
Muller *et al.* (1982)	66	59	3	11	11	19
Jensen (1985)	10	10	*	*	1	6

* Not reported.

that nutrition will be equally beneficial to both groups. This is clearly not the case as was shown recently in a large multi-centre trial; the value of nutritional support is dependent upon the level of nutritional impairment (Veterans Affairs Total Parenteral Nutrition Cooperative Study Group, 1991). Patients who are not undernourished will be subjected to the potential risks of nutritional therapy without any of the potential benefits. Of the seven trials reported, only five reported on the hospital mortality in their patients. The results were statistically significant only in the study of Muller *et al.* (1982), but the numbers of patients included in each arm of the remaining trials were insufficient to be confident that a therapeutic response had not been missed. An overview of all trials can be obtained by combining the results and calculating an odds ratio for the risk of death in all the 'fed' groups (i.e. those receiving supplements) compared with the control groups who received no supplement. From the trials reported in Table 1 the calculated odds ratio for mortality is 1·08; this is not significantly different from 1·00, which would indicate that there was no clear benefit with regards to mortality in providing peri-operative nutrition in cancer patients.

A possible explanation of these findings is that nutritional support may actually enhance tumour growth. Studies have been difficult to perform in man, but using techniques such as ultrasound and conventional radiology to measure tumours it has not been possible to demonstrate any effects of feeding on tumour growth (Nixon *et al.* 1981; Von Meyenfeldt *et al.* 1988). Based on such findings some reviewers have suggested that tumours are autologous and do not respond to the provision of nutrients (Douglas & Shaw, 1990). Animal studies, however, have demonstrated that there does appear to be a stimulation in tumour growth during feeding (Popp *et al.* 1983; Torosian *et al.* 1984; King *et al.* 1985). Although animal tumour models are often a poor representation of human cancers, it is possible that the methods used to measure tumour growth in man are insufficiently sensitive to detect any alteration in tumour size.

THE EFFECT OF FEEDING ON TUMOUR GROWTH

A more sensitive measure of tumour growth may be to measure its rate of protein synthesis, as the growth of malignant cells *in vitro* has been demonstrated to be primarily

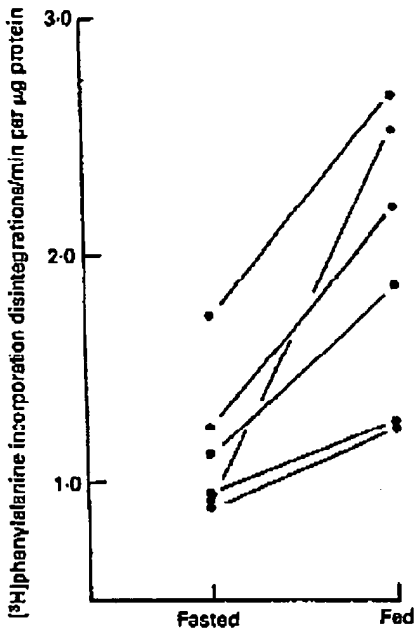

Fig. 1. Phenylalanine incorporation into tumour cells incubated in a medium enriched with 'fed' or 'fasted' serum. Each pair of linked points represents cells from one patient, incubated in that patient's own serum, collected in the fed or fasted state.

determined by the rate of protein synthesis (Lockwood *et al.* 1982; Baccino *et al.* 1984; McNurlan & Clemens, 1986). Heys *et al.* (1991) used such a technique *in vivo* to determine the effect of parenteral nutrition on colorectal cancers. It was found that the rate of protein synthesis in the tumours of patients who were fasted was 21 (range 15–35) %/d compared with 38 (range 28–61) %/d in a group of patients given 24 h of parenteral feeding. These results show that tumours certainly respond to the provision of nutrients. However, tumours are composed of a heterogeneous population of cells including cancer cells, stromal tissue cells, lymphocytes and macrophages. The consequences of an increased rate of tumour protein synthesis due to a stimulation of lymphoreticular cells are very different from a stimulation of the cancer cells themselves resulting in a stimulation of tumour protein synthesis. To determine the direct effects of feeding on cancer cells, I studied a group of six patients with histologically proven colorectal cancer. Each of these patients received an identical feeding regimen to the previous study (Heys *et al.* 1991). Before starting feeding and after 24 h of feeding blood samples were obtained for 'fasted' and 'fed' serum. The tumours were obtained at the time of surgery and by a mixture of mechanical and enzymic digestion a suspension of cancer cells was obtained (Kuppner *et al.* 1987). These cells were incubated in a medium enriched with serum from the same patient taken in either the fed or fasted state and the rate of cellular protein synthesis was determined by the incorporation of [^{3}H]phenylalanine into the cancer cells. The results are expressed as disintegrations/min (dpm) per μg protein (Fig. 1). In each case there was an increase in the rate of [^{3}H]phenylalanine uptake into the

tumour cells when incubated in the 'fed' serum, the mean increase being 78·5%. The difference between the fed and fasted groups was significant at the $P<0·02$ level (paired t test on log transformed data).

These experiments did appear to show that human cancers, in this case colorectal cancers, respond to the provision of nutrients. It was decided, therefore, to look in more detail at potential nutrients which may preferentially enhance aspects of the host defences without 'feeding' the tumour. The potential benefit of the basic amino acid, arginine, as a nutrient of this type has been suggested by its ability to reduce the growth and spread of tumours in experimental animals (Barbul, 1986). These effects appear to be as a consequence of the immune stimulatory effects of arginine in these animals (Barbul *et al.* 1977, 1980; Siato *et al.* 1987; Reynolds *et al.* 1988*a*). Similarly in man arginine has been demonstrated to enhance a number of immunological mechanisms (Barbul *et al.* 1981; Daly *et al.* 1990). In particular, arginine enhances the response of lymphocytes to polyclonal mitogens in normal volunteers. But does it have the same effect in cancer patients? Furthermore, the responses of lymphocytes to polyclonal mitogens are rather non-specific, can arginine, therefore, enhance the specific anti-tumour host defences?

IMMUNOLOGICAL EFFECTS OF L-ARGININE

Lymphocytes were obtained from the peripheral blood of six healthy volunteers and six patients with breast cancer and frozen in liquid N_2. Each subject was then given 30 g L-arginine to take orally on each of the following 3 d. On the fourth day a further blood sample was obtained and the lymphocytes separated on Ficol Hypaque gradients as described previously. The lymphocytes were again preserved in liquid N_2. After thawing the lymphocytes were incubated with phytohaemagglutinin (PHA), concanavalin A (ConA) or pokeweed mitogen (PWM) for 48 h before being pulsed with [^{3}H]thymidine and harvested onto glass-fibre discs. In each case the incorporation of the tritiated thymidine into the lymphocytes was determined by measuring radioactivity with a liquid-scintillation counter. The incorporation was determined over a range of concentrations of each mitogen and compared with controls in which lymphocytes were incubated in medium alone before pulsing with [^{3}H]thymidine. The results were expressed as stimulation indices in which the maximum thymidine incorporation was compared with the incorporation in the control medium. Fig. 2 shows the before and after arginine transformation assays for one volunteer with PHA (a), PWM (b) and (c) ConA. The responses were similar in each volunteer. Fig. 3(a) shows the stimulation indices in each of the volunteers before and after arginine for PHA stimulation; the pre-arginine stimulation index was a median of 32 (range 16–41) and after arginine a median of 61 (range 31–93). These results confirm the similar findings of Barbul (1986) and others that arginine can enhance the proliferative response of lymphocytes in healthy individuals. In the patients with breast cancer the response to PHA was most marked. The pre-arginine stimulation index was 32 (range 10–30) and this was increased to a median of 43 (range 20–71) following arginine (Fig. 3(b)). This experiment has since been repeated by Brittenden *et al.* (1992), in our laboratory, for a larger group of patients with breast cancer and their findings confirm the stimulatory effect of arginine in such patients.

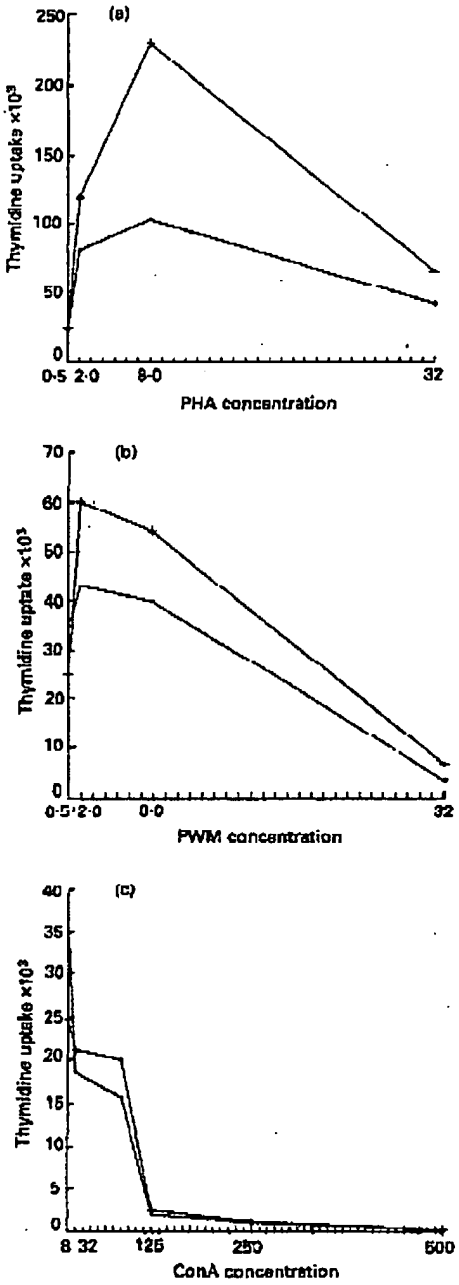

Fig. 2. Thymidine uptake of peripheral blood lymphocytes stimulated with: (a) phytohaemagglutinin (PHA), (b) pokeweed mitogen (PWM) or (c) concanavalin A (ConA) before (●) and after (+) an oral dose of arginine (30 g/d for 3 d).

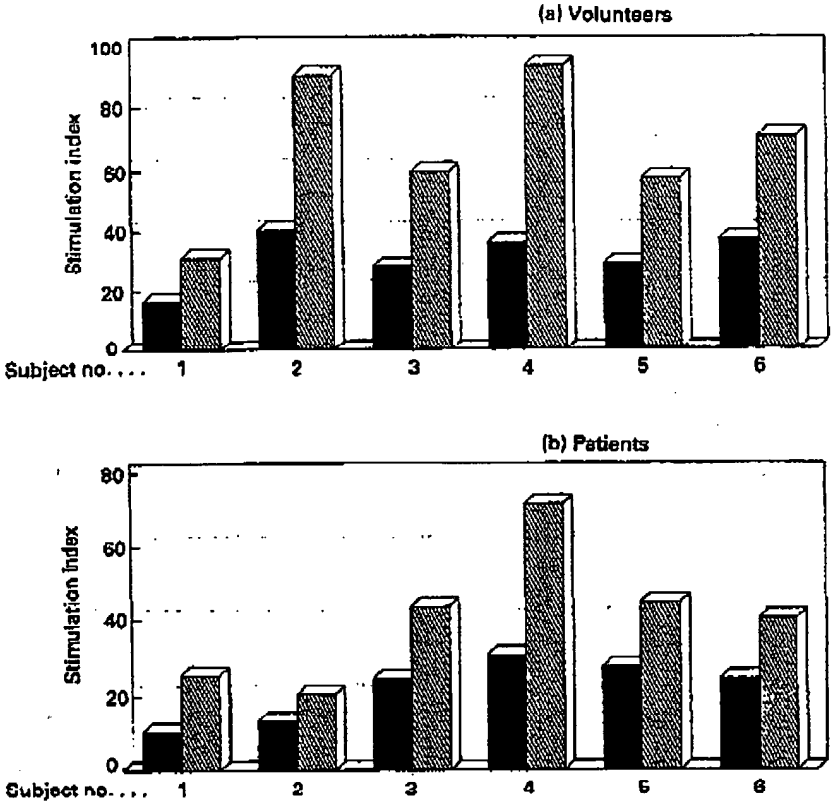

Fig. 3. Stimulation indices of mitogen transformation assays performed with phytohaemagglutinin (PHA) before (■) and after (▨) an oral dose of arginine (30 g/d for 3 d) in (a) volunteers and (b) patients with breast cancer.

The results of the previously mentioned studies are, however, rather non-specific, demonstrating only that the potential of lymphocytes to react to mitogens is increased by arginine. For arginine to be of benefit in the human host–cancer relationship it must have an effect on the effectors of specific anti-cancer immunity. As human cancers frequently do not express transplantation antigens to a high degree, human immune responses are frequently limited to the so-called natural cytotoxicity mediated by natural killer (NK) and lymphokine-activated killer (LAK) cells which are able to lyse target cells in a non-major histocompatibility complex-restricted manner and without previous sensitization to the targets (for review, see Trinchieri, 1989). The next phase of this study was, therefore, to determine the effects of arginine on human NK and LAK cell cytotoxicity. Lymphocytes were obtained from four volunteers and incubated in a medium (RPMI 1640) enriched with arginine as follows: RPMI 1640 + fetal calf serum (10 g/l) + no added arginine, RPMI 1640 + fetal calf serum (10 g/l) + 3 mmol/l arginine, RPMI 1640 + fetal calf serum (10 g/l) + 6 mmol/l arginine, RPMI 1640 + fetal calf serum (10 g/l) + 30 mmol/l arginine.

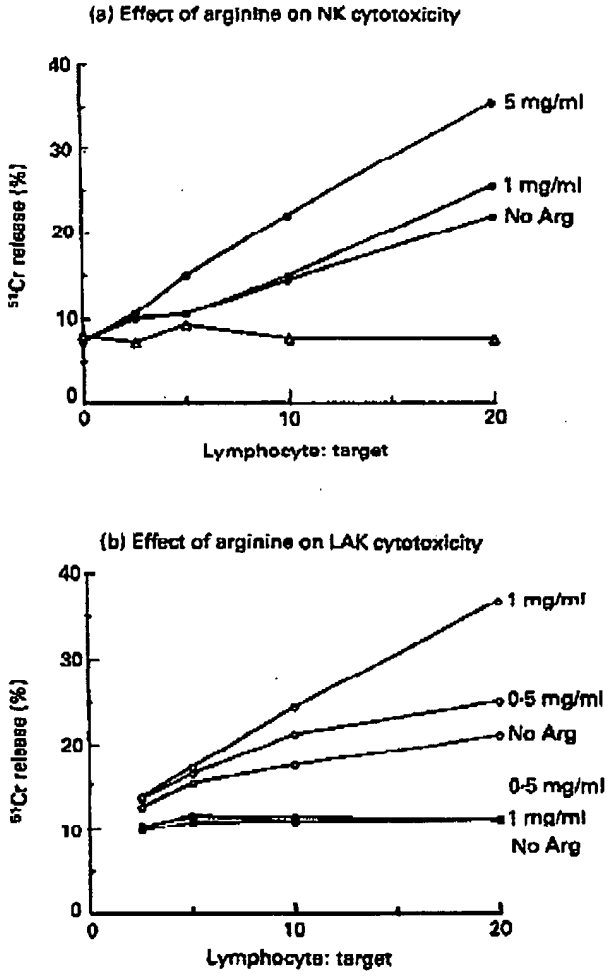

Fig. 4. The effect of arginine on (a) natural killer (NK) and (b) lymphokine-activated killer (LAK) cell cytotoxicity *in vitro*. (Δ), Lymphocytes depleted of CD56+ cells; (O), cells pre-incubated with interleukin-2; (●), cells incubated with arginine (Arg) only.

After incubation for 24 h the lymphocytes were either used as the effector cells against ^{51}Cr-labelled K562 lymphomatous cells or further incubated in medium (as described previously) + 1000 iu interleukin-2 (IL-2)/ml for 72 h before being used as the effectors against ^{51}Cr-labelled Daudi lymphomatous cells. The results of these killing assays are shown in Fig. 4. It is clear that arginine enhanced NK killing only at high concentrations; however, it appeared to enhance the response to IL-2 and, therefore, LAK cytotoxicity at relatively lower concentrations.

Table 2. *Changes in the expression of CD56 antigen on peripheral blood lymphocytes following 30 g arginine/d for 3 d*

(Values are expressed as absolute nos. of cells ($\times 10^8$) before and after arginine)

	CD56		
Subject	Pre-arginine	Post-arginine	Percentage change
1	2·0	2·4	18
2	3·0	4·4	32
3	1·4	1·5	7
4	1·6	2·8	73
5	1·3	3·4	171
6	4·8	6·3	32
7	2·2	2·6	18
8	1·3	2·9	123

If the lymphocytes were depleted of CD56+ cells before incubation then all cytotoxicity to both K562 and Daudi cells was abolished irrespective of whether arginine was added to the medium or not (Fig. 4). In contrast depletion of CD4+ or CD14+ lymphocytes did not affect the cytotoxicity.

In vivo arginine administration (30 g/d for 3 d) to volunteers was found to significantly increase the percentage, and absolute numbers of lymphocytes of the CD56+ subset (Table 2). Furthermore, in thirteen volunteers taking oral arginine, as described previously for 3 d there was a highly significant increase in the level of NK and LAK cell cytotoxicity in *in vitro* assays against K562 and Daudi cells respectively (Fig. 5). It is interesting that the two subjects with the highest baseline NK and LAK activity were the two in whom there was little or no response to arginine. In contrast the four individuals with the lowest baseline NK cytotoxicity had the greatest response to arginine; in these subjects the mean increase in cytotoxicity was 195% whereas for the whole group it was 91% for NK killing and 58% for LAK killing.

These experiments were of course conducted using volunteers. The next phase of the study was to determine the response of NK and LAK cells to arginine in cancer patients. A preliminary study was conducted in four patients with relatively advanced breast cancer; in each patient, feeding arginine (30 g/d, for 3 d) increased both NK and LAK cell activity. The present study has now been repeated with much larger numbers confirming that arginine enhances NK and LAK cell activity in cancer patients (Brittenden *et al.* 1993).

THE EFFECTS OF ARGININE ON TUMOUR GROWTH

The studies described previously have demonstrated that arginine supplementation can stimulate aspects of the host anti-tumour immune response but it is also important to determine the actions of arginine supplementation on tumour growth.

In an attempt to separate the immune stimulatory effects of arginine from the effects on a tumour a nude mouse model was used. Such animals readily accept a variety of tumour xenografts and allow the growth of these to be measured in response to a variety

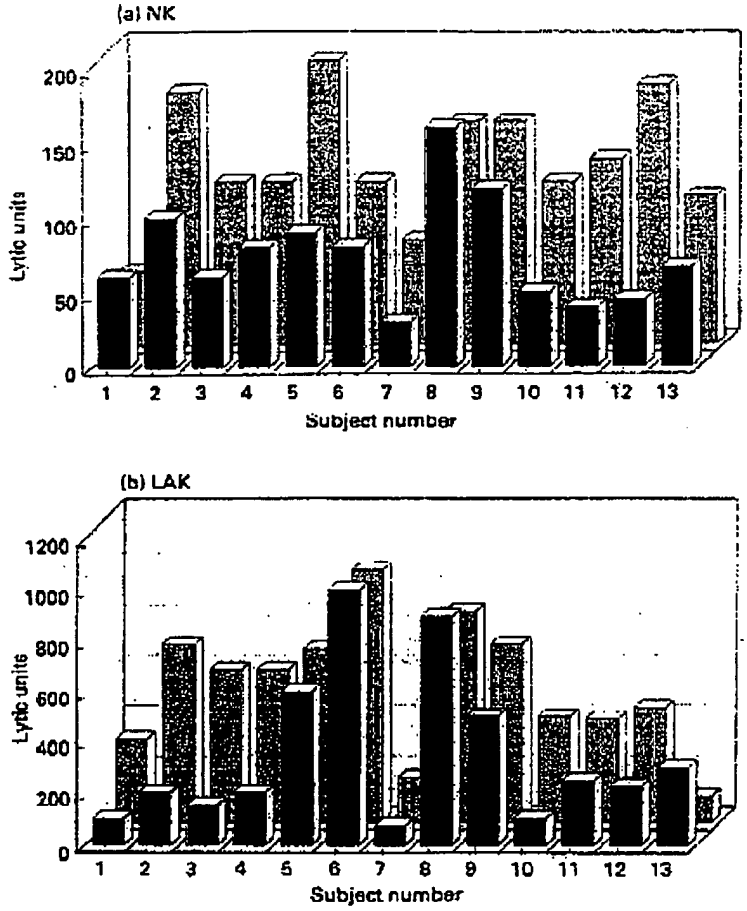

Fig. 5. Natural killer (NK) and lymphokine-activated killer (LAK) cell cytotoxicity in volunteers before (■) and after (▨) an oral dose of arginine (30 g/d for 3 d).

of treatments. In the present study MIF nu/nu mice were inoculated subcutaneously with 10^7 A549 tumour cells (a cell line originally derived from a human lung cancer). The tumours were allowed to grow for 7 d before the animals were randomized to a standard diet or an arginine-enriched diet (8 g/l in drinking water). The growth of the tumours was determined weekly with calliper measurements. After 30 d the animals were killed and the rate of tumour protein synthesis determined following the injection of a flooding dose of [^{3}H]phenylalanine (McNurlan *et al.* 1979). The tumours were also examined histologically, weighed and their protein content determined.

To our surprise it was found that the tumours of the arginine-supplemented animals had the fastest growth rates, weighed more and had higher rates of protein synthesis than the control animals (Table 3). It is interesting to note that the rates of muscle and liver protein synthesis were not dissimilar in the two groups of mice (Table 4).

Table 3. *Tumour weight, volume and protein content at 30 d compared with rates of tumour protein synthesis in MIF nude mice implanted with A549 adenocarcinoma cells*

(Mean values and standard deviations)

Treatment group	Tumour			Fractional rate of protein synthesis (%/d)
	Volume (ml)	Wt (mg)	Protein (mg)	
Control				
Mean	0·3	52·5	1·7	43·5
SD	0·14	52·5	1·1	10·9
8 g arginine/d				
Mean	0·72*	131·3*	8·4*	67·3*
SD	0·22	83·5	3·5	13·1

Mean values were significantly different from those for controls: *$P<0·05$.

Table 4. *Rates of liver, muscle and tumour protein synthesis in athymic nude mice implanted with A549 adenocarcinoma cells*

(Mean values and standard deviations)

Treatment group	Rates of protein synthesis (%/d)		
	Liver	Muscle	Tumour
Control			
Mean	71·3	1·24	43·5
SD	3·33	0·20	10·9
8 g arginine/d			
Mean	82·7	1·24	67·3
SD	5·0	0·31	13·1

These unexpected results of the animal studies began to cast some doubt on our initial hopes that arginine may be the panacea to nutritional treatment in cancer patients. It was decided, however, to carry on with an experiment to determine the effect of arginine on human tumours *in vivo*. Women with localized breast cancer were recruited into the study and randomized into two groups: group 1 received arginine supplements (30 g/d for 3 d) in addition to a standard diet, group 2 received a standard hospital diet. In both groups the rate of tumour protein synthesis was measured at the time of surgery using a 'flooding' dose technique with [1-^{13}C]leucine (4 g/70 kg body weight, 20 atoms % enrichment). The excised tumours were examined histologically and, in addition to the standard assessments of mitotic index, cellularity and tumour type, the tumours were also labelled with the monoclonal antibody Ki67. This antibody has been shown to recognize a poorly characterized nuclear antigen expressed on actively dividing cells (Gerdes *et al.* 1984). Ki67 expression has previously been shown to correlate with other assessments of cell proliferation such as *in vitro* thymidine uptake (Silvestrini *et al.* 1988; Kamel *et al.* 1989).

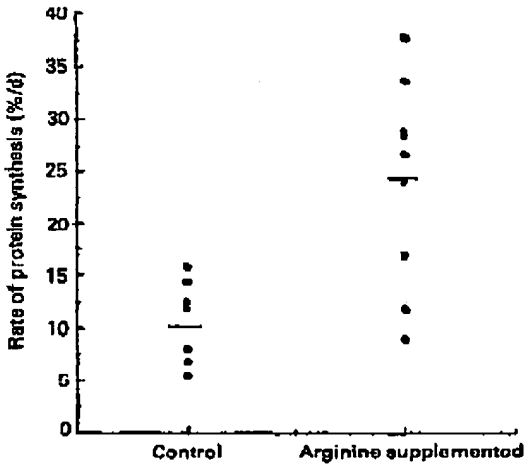

Fig. 6. Rates of tumour protein synthesis in breast cancer patients taking supplemental arginine (30 g/d) compared with controls. (—), Median values.

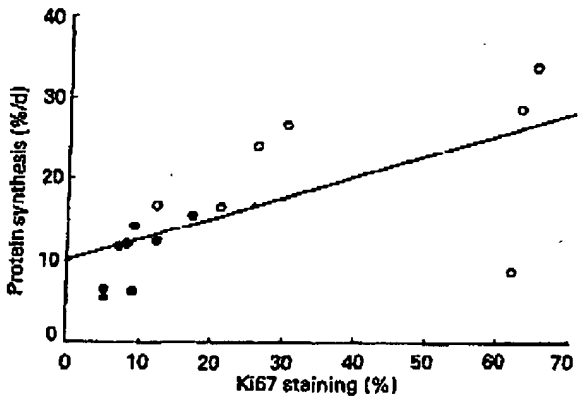

Fig. 7. Correlation between rates of tumour protein synthesis and staining with Ki67 in breast cancers in (○) controls and (●) arginine-supplemented patients.

There was no significant difference between the two groups of patients in terms of age or menopausal status. The mean plasma arginine concentration of the arginine supplemented patients was 230 (range 198–306) μmol/l compared with 160 (range 132–208) μmol/l in group 2. The insulin concentrations were similar in the two groups (Park *et al.* 1992).

The rates of tumour protein synthesis were significantly higher in the arginine-fed group of patients (Fig. 6). The median rate of protein synthesis was 25·6%/d in the

arginine-supplemented group and 10%/d in the control group. It is interesting that two patients who had high carcinoma-*in situ* components to their tumours had the two lowest rates of tumour protein synthesis. Expression of Ki67 was also higher in the arginine-supplemented patients compared with the controls and there was a close correlation between the expression of Ki67 and the fractional rate of tumour protein synthesis (r 0·78, $P<0·001$; Fig. 7). However, despite this close correlation one tumour was distant from the others on the scatter plot, with a rate of protein synthesis of 9%/d but a Ki67 expression on 62% of its cells. This tumour was one of the two which had a prominent carcinoma-*in situ* component and, therefore, was not typical of the group as a whole. The other tumour with the prominent *in situ* component was too small to obtain sufficient tissue to allow Ki67 to be assessed.

As discussed previously (Park *et al.* 1992) there was no correlation between mitotic rates or tumour grade and the fractional rates of tumour protein synthesis, but previous authors have highlighted the difficulties in such measures of tumour proliferation (Silverberg, 1976; Hall & Levison, 1990). Ki67 expression is thought to represent the proportion of the tumour within the growth cycle (Gerdes *et al.* 1984). Therefore, the present findings of a correlation between tumour protein synthesis and Ki67 expression, and a stimulation of both in breast cancers by arginine supplementation, would appear to indicate a stimulation of tumour growth by arginine.

Although L-arginine supplementation in patients with breast cancer did appear to stimulate their tumours it also stimulated the cells of the reticular endothelial system. Indeed, the measured rate of tumour protein synthesis in these experiments represents the summation of the rates of synthesis in many different cell types including: cancer cells, lymphocytes, fibroblasts and macrophages. This begs the question, rather like the colorectal tumours and total parenteral nutrition, as to which cells are responsible for the apparent stimulation of the tumours (Heys *et al.* 1991). Obviously if the rate of tumour protein synthesis and expression of Ki67 was increased due to a stimulation of tumour-infiltrating lymphocytes this would have a totally different significance from a stimulation of the cancer cells themselves. It is not possible to determine from the sections of tumours looked at in the previously mentioned study which cells were expressing Ki67. An approach, such as was used with the colorectal tumours, in which the tumours were enzymically digested and the tumour cells isolated for *in vitro* studies could not be used to determine the effects of arginine on different cell populations *in vivo*. Therefore, tumours from a further two patients given arginine were studied immunohistochemically. The rates of tumour protein synthesis in these two patients were determined as described previously, and found to be 15 and 24%/d respectively. In each case cryostat sections of the tumours were obtained and stained for Ki67 and either CD2+ lymphocytes or macrophages. Lymphocytes expressing the CD2 surface antigen include T cells and NK/LAK cytotoxic cells. In the tumour of the first patient 2591 cells were counted in ten high-power microscope fields; 12% of these cells were Ki67 positive, 20% were CD2 positive and 50% were macrophages. Only three CD2 positive cells were also positive for Ki67 and three macrophages were also positive for Ki67. In the second tumour 3433 cells were counted; 26% were Ki67 positive, 24% were CD2 positive and 30% were macrophages. Only 2% of Ki67-positive cells were either macrophages or CD2-positive lymphocytes. These results would appear to show that the observed stimulatory effects of arginine are due to direct effects on the tumour cells themselves.

ARGININE AND HUMAN TUMOURS

The results of the various studies described previously have led to the conclusion that arginine supplementation in man results in both a stimulation of the host defences and also the tumour cells themselves. These effects may occur in animals but because of the relative immunogenicity of most of these tumours the immune stimulatory effects may outweigh the tumour-enhancing effects resulting in reduced tumour growth and spread (Pryme, 1977; Tachibana *et al.* 1985; Reynolds *et al.* 1988*a*). In a poorly immunogenic mouse tumour, arginine actually enhanced tumour growth (Reynolds *et al.* 1988*b*). This is perhaps more analogous with the situation in man, where it would appear that the tumour-enhancing effects of arginine outweigh the immune stimulatory effects. Until the mechanisms for these actions of arginine are determined and, it is possible to separate the effects on tumours and the immune system the use of arginine supplementation in cancer patients must be viewed with some caution. These studies do show, however, that a single amino acid may have profound effects on human tumours and points the way to nutritional/pharmacological manipulation of tumours in man.

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